Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 645–649, February 1979
Biochemistry

Localized deposition of chitin on the yeast cell surface in response to mating pheromone

(chitin synthase/zymogen activation/asymmetric membrane assembly)

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Communicated by Clinton Ballou, November 9, 1978

ABSTRACT **Treatment of *a* mating-type *Saccharomyces cerevisiae* cells with the pheromone α-factor (secreted by α mating-type cells) induces the synthesis of chitin. Small daughter cells, which start with no detectable chitin, make 3 times more chitin when grown in the presence of α-factor than do untreated exponentially growing cells. Budding cells accumulate chitin in the nascent division septum [Cabib, E. & Bowers, B. (1975) *J. Bacteriol.* 124, 1586), as detected by staining with the fluorescent dye primulin. In the absence of a division septum, α-factor-treated cells accumulate chitin in the area of pheromone-stimulated growth. Enzymatic lysis of budding and pheromone-treated cells allows the separation of membrane-bound chitin synthase (UDP-2-acetamido-2-deoxy-D-glucose: chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) activity from a dense particulate fraction containing chitin. Chitin synthase activity is associated with both the plasma membrane and small intracellular particles. During pheromone treatment, the levels of chitin synthase in the plasma membrane and in intracellular particle fractions increase 11- and 4-fold, respectively. Although chitin synthase is made as zymogen that requires proteolytic activation, the plasma membrane of pheromone-treated cells shows a significant fraction of preactivated enzyme; intracellular membrane-bound synthase is found exclusively in the zymogen form.**

Developmental processes involve a complex temporal and spatial interplay of enzymes and cell structures. Cell surfaces, for example, have a pivotal role in providing topological asymmetry, a common feature of the differentiated state. Asymmetry implies a nonrandom distribution of membrane components, although the current paradigm of membrane structure, the fluid–mosaic model (1), suggests no long-range order. A solution to this paradox may be a cytoskeleton capable of segregating portions of the cell surface while not affecting its overall fluidity. Although results of many investigations have implied a role for cell infrastructure in the maintenance of surface asymmetry, few experimental systems provide an opportunity for detailed molecular analysis. The insertion of chitin specifically into the yeast division septum is one favorable example.

Cabib and Farkas (2) have shown that chitin synthesis is temporally and spatially regulated during the budding cycle in *Saccharomyces cerevisiae*. Chitin, which represents only 1–2% of the yeast cell wall carbohydrate, is located in division septa and bud scars. As anticipated from this distribution, maximal synthesis of chitin occurs at the time of septum closure. Chitin synthase (UDP-1-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16) is a plasma membrane enzyme that, in budding cells, is found largely in a zymogen form requiring proteolytic activation (3), perhaps by a protease found in the yeast vacuole. Cabib *et al.* (4) proposed that the vacuole recognizes and interacts with a septum-limited fraction of the synthase zymogen molecules, thus leading to local activation and chitin synthesis.

In this paper we describe another instance in which yeast cells regulate the activation of chitin synthase. We demonstrate that the yeast mating pheromone, α-factor, increases the rate of synthesis of chitin, increases the rate of synthesis of chitin synthase zymogen, and leads to activation of a plasma membrane fraction of the synthase, all of which result in a localized deposition of chitin on the cell surface.

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MATERIALS AND METHODS

Materials. *S. cerevisiae* haploid strains X2180-1A (*a*) and 1B (α) were obtained from the Yeast Genetics Stock Center. High acid phosphatase-secreting A279 [*a*/α (II ACP 1-2/II ACP 1-2)/(U ACP 1-2, P/U ACP 1-2, P)], obtained from P. Hansche (University of California, Davis), was sporulated and an *a* haploid strain, called A279-2c, was selected. Cells were grown in YPD medium which contains 1% Bacto-Yeast extract, 2% Bacto-Peptone, and 2% glucose.

Lyticase is a highly purified lytic β-1,3-glucanase isolated from the culture fluid of *Oerskovia xanthineolytica* cells grown on insoluble glucan (unpublished data). Fraction II (30,000 units/mg; 1 unit will lyse 0.2 OD_{600} unit of logarithmic phase *S. cerevisiae* in 30 min at 30°C) was used. α-Factor was purified from the culture fluid of X2180-1B cells as described by Ciejek *et al.* (5), and the phosphocellulose fraction (≈15,600 units/mg; 1 unit will arrest budding in 10^5 X2180-1A cells for 3 hr) was used without further purification. Protease B inhibitor was purified from stationary phase X2180-1A to the DEAE-cellulose fraction (5.4 units/mg) as described by Cabib (6).

Other reagents were obtained as indicated: Ficoll, sorbitol, GlcNAc, UDP-GlcNAc, *p*-nitrophenylphosphate, trypsin, and lactoperoxidase were from Sigma; $Na^{125}I$ and chitinase were from ICN; Glusulase was from Endo Laboratories (Garden City, NJ); Renografin was from Squibb; primulin was from Tridom/Fluka (Hauppauge, NY); UDP-[6-^{3}H]GlcNAc (6.6 Ci/mmol) was from New England Nuclear.

Chitin Estimation and Localization. (*i*) *Isolation and α-factor treatment of newborn cells.* The last division event before yeast cells enter stationary phase produces a small daughter cell and a normal-size parent cell (7); differential centrifugation was used to prepare a fraction highly enriched in such small daughter cells. X2180-1A cells, grown to stationary phase ($OD_{600} \approx 35$), were concentrated 3- to 4-fold by centrifugation and resuspended in 0.05 M potassium phosphate (pH 7.0). The cell suspension, in 12-ml aliquots, was sonicated for 10 sec at setting 3 (50 W) with the microtip probe of a model W185D cell disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY) and then centrifuged at 300 × *g* for 3 min. The pellet was reextracted and centrifuged a total of three times. The pooled supernatant fraction was centrifuged at low speed once to remove the remaining large cells, and the small daughter cells

were isolated by sedimentation at 10,000 × *g* for 10 min. Cells were resuspended in growth medium to an OD_{600} of 0.2–0.5, and α-factor (50 units/ml) was added where indicated. Aliquots were withdrawn at intervals, and the cells were harvested and stored on ice. Additional α-factor was added at 3 hr.

(*ii*) *Extraction and enzymatic digestion of cell walls.* Cell wall ghosts were prepared by alkaline extraction (8). Chitin was hydrolyzed in a mixture containing 2 mg of cell ghosts, 34 units of chitinase (67 units/mg measured on colloidal chitin; 1 unit will release 1 nmol of GlcNAc per min), and 0.05 M sodium succinate (pH 6.3) in a volume of 0.25 ml. Incubations for 1 hr at 37°C were terminated by boiling for 3 min. Glusulase [50 μl; chromatographed on Sephadex G-25, 37 mg/ml (9)] was added to centrifuged hydrolysates, and the incubation was continued for 30 min at 30°C. The reactions were diluted 1:2 with succinate buffer and boiled for 3 min, and GlcNAc was determined by the method of Reissig *et al.* (9).

(*iii*) *Fluorescence and phase-contrast microscopy.* Cells were treated with primulin (1.0 mg/ml) in buffer, centrifuged, washed, and examined with a Zeiss Photomicroscope III equipped with an epifluorescence condensor. Tri-X film was used for phase-contrast and fluorescence photomicroscopy.

Membrane Isolation. (*i*) *Spheroplast preparation and lysis.* Cells grown to the indicated OD_{600} were centrifuged, washed with 0.05 M potassium phosphate (pH 7.5), and resuspended in a minimal volume at 0°C. Cells were then diluted to an OD_{600} of 200 in 1.4 M sorbitol/0.02 M potassium phosphate, pH 7.5/20 mM 2-mercaptoethanol containing 25–50 units of lyticase fraction II per 1 OD_{600} of cells. After 30 min at 30°C, the spheroplasts were sedimented at 10,000 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended with a Dounce homogenizer in one-fifth volume of lysis buffer (5% Ficoll/0.1 M sorbitol/0.05 M potassium phosphate, pH 6.5/1 mM sodium azide). After 10 min at 0°C, the extract was centrifuged at 10,000 × *g* for 5 min, the supernatant was collected, and the pellet was reextracted with lysis buffer. After another centrifugation, the supernatant fractions were pooled (low-speed supernatant), and the pellet (low-speed pellet) was resuspended as indicated.

(*ii*) *Sucrose step-gradient equilibrium sedimentation.* The low-speed pellet fraction was resuspended by homogenization in 2.5 or 17 ml of 45% sucrose/0.05 M potassium phosphate, pH 6.5. This mixture was layered on top of an equal volume of 60% sucrose in the same buffer in a cellulose nitrate ultracentrifuge tube. Step-gradients were centrifuged at 100,000 × *g* for 90 min at 4°C in the Beckman SW 50 or SW 27 rotor and model L or L2-65B ultracentrifuge. The following fractions were withdrawn and diluted separately with 5–20 ml of lysis buffer: 45% sucrose phase, 45–60% sucrose interface, and 60% pellet. The diluted fractions were centrifuged at 27,000 × *g* for 10 min, the supernatants were discarded, and the pellets were resuspended in a minimal volume of lysis buffer.

(*iii*) *Renografin equilibrium sedimentation.* Fractions were layered on top of linear 7–35% Renografin gradients in 0.1 M sorbitol/0.02 M sodium citrate, pH 6.5. Gradients were centrifuged in the Beckman SW 27 or 27.1 rotor at 25,000 rpm for 2.5–5 hr at 4°C, and fractions were collected from the top with a Searle/Buchler auto Densi-Flow IIc. Fractions were diluted with 2–3 vol of lysis buffer, and the membranes were collected by centrifugation at 110,000 × *g* (type 40 rotor) for 90 min. Membrane pellets were resuspended in 0.2 ml of lysis buffer.

Enzyme Assays and Other Methods. Chitin synthase was assayed as described by Cabib (6). Radioactive product was monitored by filtration on GF/C filters. Total zymogen was determined by a single incubation in which 0.5–2 μg of trypsin was used to activate the enzyme in the presence of substrates. In assays of preactivated enzyme, the trypsin was omitted and reaction mixtures were supplemented with 0.15 unit (6) of protease B inhibitor. Addition of protease B inhibitor to fractions during membrane isolation did not diminish the preactivated enzyme. One unit of synthase will incorporate 1 nmol of

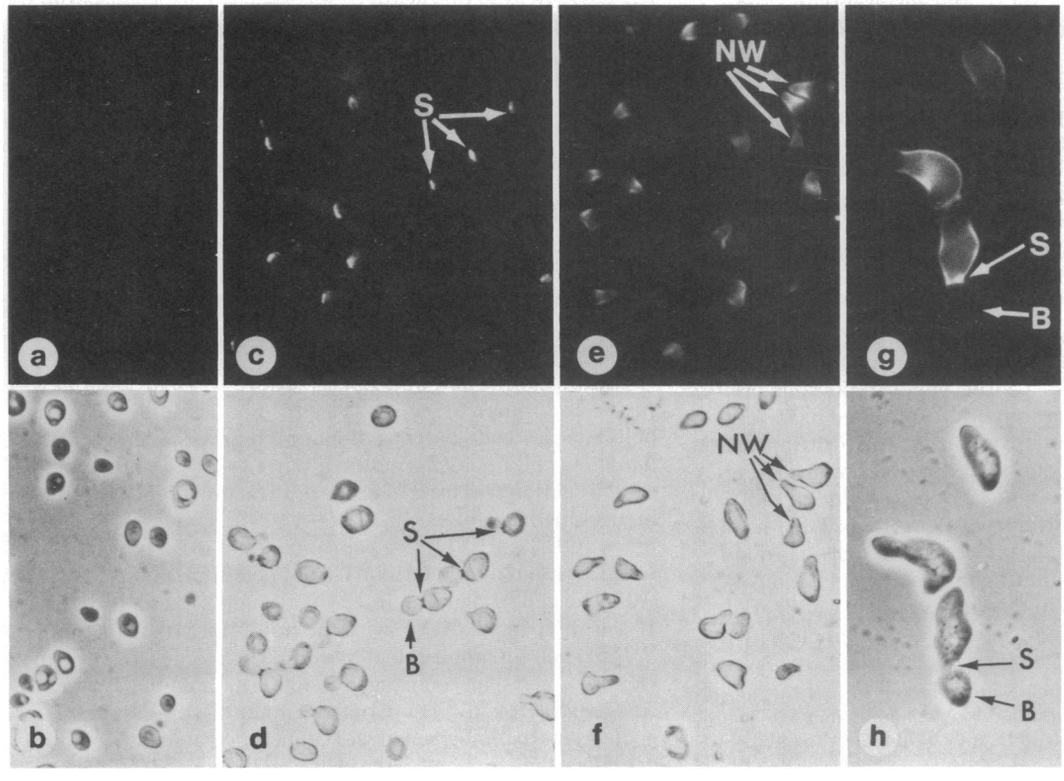

FIG. 1. Fluorescence (*Upper*) and phase-contrast (*Lower*) microscopy of primulin-stained small daughter cells (*a* and *b*), exponential phase cells (*c* and *d*), and cells treated with α-factor for 3 hr (*e* and *f*) or 7 hr (*g* and *h*). After 7 hr, α-factor was removed and budding growth reinitiated. B, bud; S, septum; NW, new wall. (×320.)

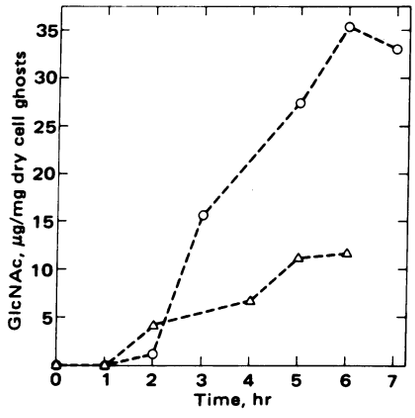

FIG. 2. Chitin content of small daughter cells grown with (○) and without (△) α-factor. One milligram of dry cell wall ghost corresponds to 50–70 OD_{600} units. △, Budding cell chitin content; ○, α-factor-treated cell chitin content.

GlcNAc into chitin per min at 30°C. Acid phosphatase was assayed (10) with *p*-nitrophenylphosphate as substrate; 1 unit will release 1 nmol of *p*-nitrophenol per min. Iodination of spheroplasts with ^{125}I and lactoperoxidase was performed as described by Duran *et al.* (3). The optical density of cell suspensions was measured in a 1-cm quartz cuvette at 600 nm in a Zeiss PMQ II spectrophotometer; 1 OD_{600} unit corresponds to about 10^7 cells. Protein concentration was determined by the Lowry method. Alkali-insoluble glucan was measured by the phenol/sulfuric acid method with glucose as the standard (11). Radioactivity was measured in a Searle Delta 300 liquid scintillation counter for ^3H and in a Searle 1185 gamma counter for ^{125}I.

RESULTS

α-Factor-Treated Cells Incorporate Chitin into the New Growth Zone. Small daughter yeast cells contain much less chitin than is found in exponentially growing cells (7). When the small daughter cells were allowed to grow in fresh medium, primulin-staining material accumulated at the parent–bud junction that formed (Fig. 1 *a–d*). Cabib and Bowers (12) have shown that this staining is chitin-specific in *S. cerevisiae*. Small daughter *a* mating-type cells did not form buds when treated with α-factor; instead, the cells showed the well-known polar elongation (Fig. 1*f*; ref. 13). Such cells showed fluorescence on the area of new growth when stained with primulin (Fig. 1*e*). After α-factor was removed from the culture, new buds appeared at the growing pole (Fig. 1*h*), and these new buds did not stain with primulin (Fig. 1*g*).

Most of the GlcNAc was released from alkali-extracted cell wall chitin preparations by chitinase; longer incubations or addition of more chitinase did not release more GlcNAc. α-Factor-treated *a* cells accumulated up to 3-fold more chitin than did exponentially growing cells (Fig. 2). In contrast, when treated with excess α-factor for 6 hr, α mating-type small daughter cells grew exponentially and had only 14 μg of GlcNAc per mg of dry cell wall ghost. Thus, the α-factor effect on chitin synthesis is mating-type specific.

Chitin Synthase Activity and Cell Wall Carbohydrate Are Separable. A purified lytic β-1,3-glucanase preparation forms osmotically sensitive spheroplasts of *S. cerevisiae* (unpublished data). When spheroplasts of budding or α-factor-treated cells were lysed in a hypotonic buffer, 60% of the chitin synthase activity sedimented at low speed (5×10^4 *g*·min). This rapidly sedimenting fraction contained 21–28% of the chitin found in an equal amount of alkali-extracted cell ghosts (Table 1) but only 0.2–0.3% of the alkali-insoluble carbohydrate measured by the phenol/sulfuric acid method (Table 1). The chitin synthase activity and chitin were further resolved by equilibrium density sedimentation on a sucrose step-gradient. Of the chitin synthase activity recovered from the gradient, 75–80% sedimented at the 45–60% sucrose interface, whereas >90% of the chitin sedimented through 60% sucrose. Thus, the synthase and its product are readily separated. A low level of chitinase (22.9 units/mg) contaminating the lytic enzyme fraction probably accounts for the incomplete recovery of chitin.

Two Membrane Fractions Contain Chitin Synthase. The chitin synthase activity that was not sedimented at low speed from a lysate (40%) required a higher speed (6×10^6 *g*·min) for sedimentation. The low-speed sedimentable fraction was identified as plasma membrane by coincident purification of chitin synthase activity and ^{125}I label that was catalytically attached to spheroplasts prior to lysis (3). Fig. 3*B* shows a Renografin density gradient profile of plasma membrane in which ^{125}I label and chitin synthase activity sedimented coincidently ($\rho = 1.16$).

The low-speed supernatant activity showed a broad bimodal density profile when sedimented on a Renografin gradient (Fig. 3*A*, $\rho = 1.11$–1.19), and about 7% of the ^{125}I-labeled plasma membrane was present in this fraction. The chitin synthase in this fraction sedimented with particles containing the secreted glycoprotein acid phosphatase.

α-Factor Induces the Synthesis and Activation of Chitin Synthase. Treatment of *a* and α mating-type small daughter cells with α-factor led to an increase in the total chitin synthase activity in extracts of *a* cells only. This increase in activity was seen in both the plasma membrane fraction (Fig. 4*A*) and the slowly sedimenting intracellular particles (microsomal fraction,

Table 1. Separation of membrane-bound chitin synthase activity from cell wall carbohydrate

	Budding cells			α-Factor-treated cells		
	Chitin synthase, units	Chitin, mg	Alkali-insoluble carbohydrate, mg	Chitin synthase, units	Chitin, mg	Alkali-insoluble carbohydrate, mg
Cells	—	1.07	18.90	—	2.84	36.40
Extract	10.5	0.30	ND	30.6	0.58	ND
Low-speed sediment	6.1	0.18	0.06	17.9	0.34	0.09
45% sucrose phase	0.1	<0.01	ND	1.6	0.02	ND
45–60% sucrose interface	3.2	<0.01	ND	11.3	<0.01	ND
60% sucrose pellet	0.7	0.15	ND	2.2	0.30	ND

X2180-1A cells [1400 OD_{600} units untreated or treated with α-factor (50 units/ml) for 2 hr] were converted to spheroplasts with 25 units of lyticase per OD_{600} (for budding cells) or 50 units of lyticase per OD_{600} (for α-factor-treated cells). Chitin and carbohydrate determinations were performed on material extracted once with alkali (8). ND, not determined.

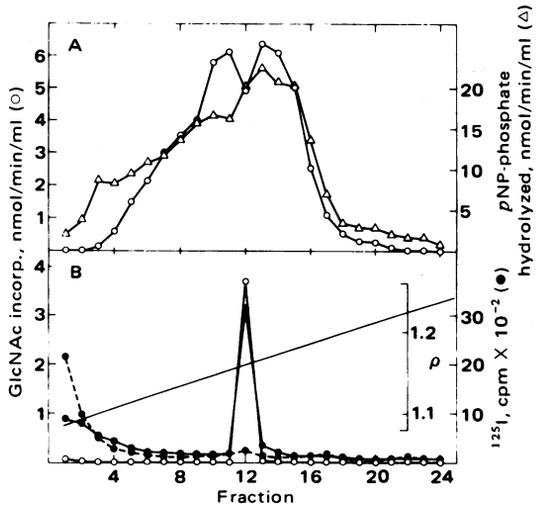

FIG. 3. Equilibrium sedimentation of intracellular and plasma membrane. (*A*) Cells (3000 OD_{600} units) from a late logarithmic-phase culture (OD_{600} = 26) of A279-2c were lysed with lyticase fraction II (75,000 units). The low-speed supernatant fraction (5 ml) was applied directly to a 33-ml Renografin gradient, centrifuged for 5 hr, and processed. Of 31 units of chitin synthetase (○) in the extract, 14 units from the low-speed supernatant fraction was sedimented at high speed (6 × 10^6 *g*·min), and 11 units was recovered from the gradient. Of 305 units of acid phosphatase (△) in the extract, 47 units from the low-speed supernatant fraction was sedimented at high speed, and all of this was recovered from the gradient. (*B*) In a separate experiment, spheroplasts from 670 OD_{600} units of cells were prepared. One-half of the sample was iodinated with 3.3 μCi of Na^{125}I and lactoperoxidase (3). Both samples were harvested, lysed, and fractionated. The low-speed supernatant fractions and the 45–60% sucrose interface fractions were sedimented on 16-ml Renografin gradients for 2.5 hr. ●, ^{125}I from the extract of labeled spheroplasts; dashed line, low-speed supernatant fraction; solid line, sucrose interface fraction. Of 2.3 units of chitin synthase (○) activity from the unlabeled extract, 1.2 units sedimented at low-speed, 1.1 units was in the sucrose interface fraction, and 0.7 unit was recovered from the Renografin gradient. ○, Chitin synthase activity; ●, ^{125}I cpm; △, acid phosphatase activity.

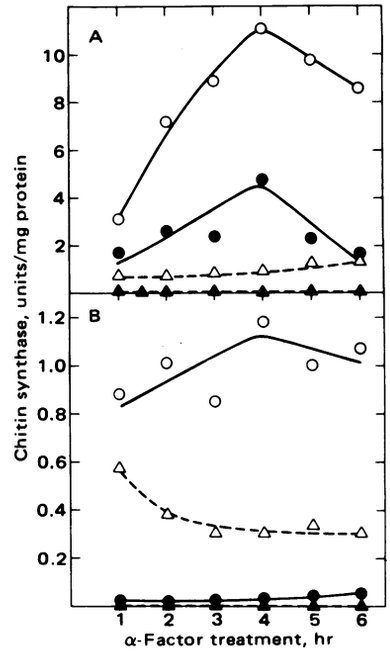

FIG. 4. α-Factor induction of the synthesis and activation of chitin synthase. (*A*) Plasma membrane fraction; (*B*) microsomal fraction. Small daughter X2180-1A (*a*) (○, ●) and 1B (α) (△, ▲) cells were grown with α-factor (50 units/ml) and aliquots were withdrawn at 1-hr intervals. Cell samples (50 OD_{600} units each) were lysed (1250 units of lyticase), the low-speed pellet fractions were purified, and the low-speed supernatant fractions were concentrated by centrifugation at 110,000 × *g* for 90 min. The sucrose interface fractions (*A*) and the concentrated supernatants (*B*) were each resuspended in 0.1 ml of lysis buffer and assayed for chitin synthase, with (○, △) and without (●, ▲) trypsin treatment.

Fig. 4*B*). After a 4-hr treatment with α-factor, the plasma membrane fractions from *a* and α small daughter cells had chitin synthase activities of 11.1 and 1.0 unit/mg of protein, respectively; the corresponding microsomal fractions had 1.2 and 0.3 unit/mg of protein.

In addition to an increase in total chitin synthase activity, α-factor treatment of *a* cells led to an increase in activity that was expressed in the absence of trypsin activation (Fig. 4). In the 4-hr sample described above, the plasma membrane fraction from α-factor-treated *a* cells had preactivated chitin synthase that was 43% of the trypsin-stimulated level (4.8 units/mg of protein); the corresponding α cell plasma membrane fraction was only 6% active without trypsin (0.06 unit/mg protein). The preactivated synthase was found only in the plasma membrane fraction; less than 5% of the total microsomal activity was detected in the absence of trypsin.

DISCUSSION

Haploid *S. cerevisiae* cells of one mating type initiate a defined developmental program in response to cells of the opposite mating type. The soluble mating pheromone α-factor interrupts *a* mating-type cells early in the cell cycle and produces polar growth without budding (13). We have shown that chitin, which is largely restricted to the division septum during budding growth, is deposited over a much larger cell surface area formed in the presence of α-factor (Figs. 1 and 2). Other cell surface-related effects of α-factor have been noted. Lipke *et al.* (14) demonstrated formation of a surface fuzzy coat and an increase in the ratio of glucan to mannan, with a decreased average chain length of the latter polymer, whereas fluorescent concanavalin A showed enhanced labeling of the new cell surface formed in the presence of α-factor (15).

It is not clear from our results whether chitin participates in yeast mating. During zygosis, mating cells contact each other at the point of growth, the same area occupied by chitin on *a* mating-type cells during α-factor treatment. Chitin could serve to anchor mating cell adhesion and fusion factors, or a patch of chitin, enriched in mating factors, could limit the number of cells in a productive union. The cytoplasmic membrane appears to be a focal point for localized chitin synthesis, because most of the chitin synthase zymogen is associated with it (Fig. 3*B*; ref. 3). The increased rate of chitin synthesis during α-factor treatment is accomplished by an increase in the amount of synthase and by the conversion of as much as one-half of the zymogen to an active form (Fig. 4).

How is the zymogen delivered to the cell surface? In common with other eukaryotic cells (16, 17), yeast cells may use a membranous vehicle, such as a secretory vesicle, for the insertion of new membrane components and for secretion of extracytoplasmic glycoproteins. Such vesicles have been identified in yeast cell thin sections: during exponential growth they are grouped under the developing bud (18); at cytokinesis they congregate at the division septum (18); and during zygote formation they are found under the growing cell tip (19). Appropriate markers for such vesicles include chitin synthase, representing a membrane component, and the secreted enzyme acid phosphatase (20). These secretory vesicles may be repre-

sented in the slowly sedimenting fraction of intracellular chitin synthase described in this report (Fig. 3A). When displayed on a Renografin density gradient, acid phosphatase and chitin synthase sedimented together. Further fractionation will be required to determine if these markers are contained within the same structure or are associated with distinct particles.

Other candidates for the secretory role have been suggested (21, 22), such as small membranes related to the vacuole. It was proposed that the plasma membrane may be derived from the vacuole by way of low-density vesicles (22). Cabib *et al.* (23) have shown, however, that chitin synthase is not associated with the vacuole; this enzyme, and perhaps others, is carried by another vesicle. Bartnicki-Garcia and colleagues (24, 25) have suggested that specialized intracellular particles, called chitosomes, deliver chitin synthase zymogen to the cell surface in *Mucor rouxii*. These chitosomes may correspond to the secretory vesicles described here.

How is local zymogen activation achieved? Cabib and Bowers (8, 12) have shown that, during budding, chitin synthase activation is restricted to a small region of the cell surface. We have demonstrated that activation induced by α-factor covers a larger region. We also observe that active synthase is restricted to the plasma membrane, whereas synthase associated with intracellular membranes is exclusively in the zymogen form (Fig. 4*B*). Newly synthesized zymogen may be transported to the plasma membrane in association with secretory vesicles, and zymogen activation could then occur concomitant with or subsequent to vesicle–cell surface fusion. Secretion occurs at the growing end of a prezygotic cell. When α-factor treated cells are derepressed for acid phosphatase synthesis, secreted enzyme (detected by cytochemical staining) is restricted to the area of new growth (unpublished data). This suggests that new chitin synthase zymogen may also be inserted into a restricted patch of the cell surface.

Cabib *et al.* (4) have proposed that activation of chitin synthase is achieved by local introduction of a vacuolar protease at appropriate sites on the inner surface of the plasma membrane. Other elements, in addition to zymogen, must serve to direct the activating machinery. During budding, a structure composed of filamentous rings is seen apposed to the inner membrane surface of the nascent division septum (26), the same area where chitin synthesis occurs. These filaments may regulate local zymogen activation during cell division or α-factor-stimulated growth.

Two other examples involving altered chitin distribution were reported recently. In the dimorphic yeast *Candida albicans*, the transition from budding to hyphal growth is accompanied by an increased rate of chitin synthesis and a shift in the location of chitin deposition from the division septum to the hyphal tip (27). Sloat and Pringle (28) have examined the distribution of chitin on the surface of a conditionally lethal *S. cerevisiae* cell division mutant (*cdc* 24; ref. 29) that fails to bud at a nonpermissive temperature but grows over the whole surface. This mutant deposits chitin nearly uniformly over the enlarging cell surface instead of forming the characteristic septum ring. The *cdc* 24 gene product may play a pivotal role in the initiation and maintenance of asymmetric yeast growth.

We thank Janet Scott for assistance in vesicle isolation, Charles Field for help in photography, and Clint Ballou for training and use of the Zeiss Photomicroscope III. This work was supported in part by grants from The University of California Cancer Research Committee, the University of California Biomedical Research Support Fund, the California Division of the American Cancer Society, and the National Science Foundation. V.B. was a University of California President's Undergraduate Fellow.

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